Modified Resazurin Test for Estimating Bacterial Counts in Maple Sap: Color Standards

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An end point color standard based on reflectance, which can be described by the Munsell system of color notation, was developed for the modified resazurin test used to estimate bacterial counts in maple sap. In order to secure a reflected color, a sterile, nonfat milk solution was added to the clear sap-dye test solution to render it opaque. Results of tests made in accordance with this method are presented in graphic form.

In accordance with the recommendations made in the 1970 report of the Associate Referee (1), a study was initiated to develop a standard color for the end point of the modified resazurin test for estimating the bacterial population in raw maple sap. Prior to this study, all testing of the method had been done by one technician and no attempt had been made to describe an end point color. Existing color standards for resazurin reduction tests used in the dairy industry are based on the reflectance of color from an opaque (skim or whole milk) solution to which the dye has been added. The reflected color for this end point is described in terms of the Munsell system of color notation (2).

Preliminary investigations of color standards for the resazurin test on maple sap indicated that suitable standards might be secured either by changing the media used in the test to present an opaque solution, which would permit the end point to be described in terms of the Munsell system, or by developing a transparent color comparator system. Experience at this laboratory in developing permanent standard amber colors for maple sirup indicated that the expense of the latter approach to this problem would be prohibitive. Moreover, the dichroism of resazurin dye in a clear solution would have made the development of such a standard exceedingly difficult. Therefore, it was decided to revise the method by devising an additive for the sap-dye test solution that would render the solution opaque and permit the end point to be read as a reflected color capable of being expressed in terms of the Munsell standard system of color notation.

Preliminary studies were made in which white blotting paper, filter paper, or absorbent cotton, respectively, was placed in test tubes and sterilized before the test materials were added to the tubes. None of these materials proved to be suitable for end point color determination because they could not provide a continuous smooth-textured surface for color reflectance from the test tubes. It was apparent that a revision of the method to provide an opaque test solution was a prerequisite for the establishment of an end point color which could be adequately described in terms of existing standard colors. The revised method is given below.

METHOD

Apparatus and Reagents

(a) *Serological pipets.*—To deliver 1 and 10 ml, with 1.0 ml graduations (sterilized).

(b) *Test tubes.*—150 × 16 mm, screw-top with molded plastic caps (sterilized).

(c) *Incubator or water bath with opaque cover.*—Constant temperature, capable of holding temperature at 37.5±0.5°C.

(d) *Bottles.*—200 ml amber, glass-stoppered.

(e) *Nonfat milk solution.*—Dissolve 100 g instant nonfat dry milk in 500 ml distilled water. Sterilize by autoclaving 15 min at 15 psig.

(f) *Resazurin dye.*—Place 200 ml distilled water in amber glass bottle and sterilize by autoclaving 15 min at 15 psig. Using sterile, dry forceps, add 1 standard resazurin dye tablet and shake to insure complete solution of dye before water cools. Store in cool dark place. Prepare fresh weekly.

Procedure

To sterile test tube, transfer 1 ml nonfat milk solution and 10 ml sap. Mix by capping and inverting tube. Incubate tube 30 min at 37.5°C. Remove tube from incubator and, with sterile pipet, add 1 ml

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resazurin dye. Cap tube, invert to mix dye thoroughly, and incubate at 37.5°C. Examine tube for color change to bluish purple standard P 6/8 end point, and note time of end point. Do not agitate tube before making reading. Estimate bacterial cell population in sap from curve relating time of color development to cell count (Fig. 1).

Results and Recommendation

Conventional pour plate techniques were used in performing all bacterial counts in this study; tryptone glucose extract agar (Difco) was used as the culture medium. The sap-milk mixture (control) exhibited a purplish purple-blue color which matched Munsell standard PB 6/10. As the resazurin due was reduced, the test solution color (expressed as Munsell standards) changed successively to bluish purple P 6/8, purplish red-purple RP 5/6, and reddish red-purple RP 6/10. The P 6/8 standard was selected as a suitable end point color for this test because it was the first sharp color change to be observed in the sequence of colors which were noted as the resazurin was reduced to resorufin.

Figure 1 shows a curve in which the bacterial populations in raw maple sap samples were plotted vs. the time required for these microorganisms to reduce the resazurin dye to the P 6/8 end point color by this method. The solid line was constructed from results obtained from 82 determinations and was fitted by the least squares method. The interrupted lines delineate 99% confidence limits. An analysis of variance of these data showed a significant F-value ($p = 0.01$). The end point color change was achieved within 1 hr by bacterial populations in the 10^7 cells/ml range. Therefore, with the use of this test, a highly contaminated lot of sap could be identified within 1½ hr after its delivery at a maple sap evaporator plant. The addition of nonfat milk solution to the sap enables the technician to secure a sharp end point color without the confusing dichroism encountered when clear test solutions are used.

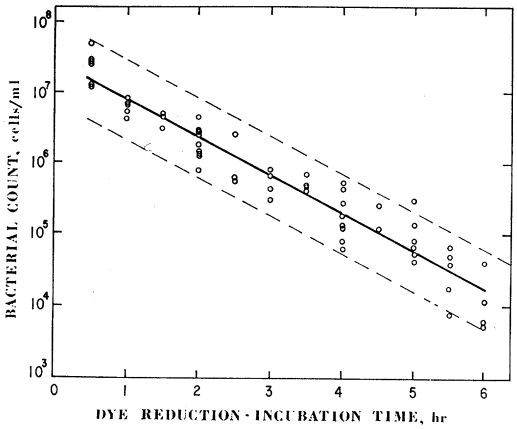

FIG. 1—Relation of bacterial population in maple sap to resazurin dye reduction time to obtain P 6/8 end point color in maple sap with nonfat milk additive.

It is recommended that study of this method be continued by subjecting the method to collaborative study.

Acknowledgments

The Associate Referee acknowledges the assistance of Virginia M. Metzger, mathematician, and R. A. Bell, chemist, both of USDA, in carrying out the work reported here.

References

(1) Kissinger, J. C. (1970) *JAOAC* **54**, 27–29

(2) Walter, W. C. (ed.) (1967) *Standard Methods for the Examination of Dairy Products*, 12th ed., American Public Health Association, New York

This report of the Associate Referee was presented at the 85th Annual Meeting of the AOAC, Oct. 11–14, 1971, at Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was accepted by the Association; their reports will appear in *JAOAC* **55** (March 1972).